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Photochemical Enzyme Co-Factor Regeneration: Towards Continuous Glutamate Monitoring with a Sol-Gel Optical Biosensor

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ABSTRACT

Sol-gel encapsulation has recently surfaced as a successful approach to biomolecule immobilization. Proteins, including enzymes, are trapped in the pores of the sol-gel derived glass while retaining their spectroscopic properties and biological activity. Our current work extends the unique capabilities of biomolecule-doped sol-gel materials to the detection of glutamate, the major excitatory neurotransmitter in the central nervous system. We are developing an *in vivo* fiber optic biosensor for glutamate along with methods to achieve continuous monitoring. In our research to date we have encapsulated GDH in a silica sol-gel film on the tip of an optical fiber. GDH catalyzes the oxidative deamination of glutamate to α-ketoglutarate and the simultaneous reduction of NAD^+ to NADH. To quantify the glutamate concentration, we observe the rate of change of NADH fluorescence as a function of time. An important consideration for continuous *in vivo* monitoring is the incorporation of a self-sustaining NAD^+ source. We have adopted a photochemical means of regenerating NAD^+ from NADH, by irradiating thionine (3,7-diaminophenothiazin-5-ium) which we incorporate into the sol-gel sensor material. When excited with visible light ($\lambda_{abs} \sim 596$ nm), thionine undergoes a reaction with NADH resulting in a non-fluorescent form of thionine and NAD^+. We have characterized the kinetics of this reaction in the sol-gel matrix, and have shown that the reaction results in regenerated co-factor that is usable by GDH for the oxidation of glutamate.

INTRODUCTION

Glutamate is the most prominent excitatory neurotransmitter in the central nervous system. It is present throughout the entire brain, but acts locally to produce different types of signals that vary in their spatial and temporal characteristics. Glutamate signaling is an important component of networks involved in attention, learning and memory, and motor control. Current detection techniques are limited in their ability to achieve the required resolution to observe these signals. New types of sensors are needed; optical sol-gel sensors could help to fill the gap.

Enzymes are commonly employed in biosensors because they provide both biochemical recognition and transduction of the recognition event into a reaction. Redox enzymes in particular are convenient because the electron transfer provides a measurable current or a measurable change in the spectroscopic properties of the substrate or co-factor. Dehydrogenases are the largest class of redox enzymes, but their requirement of an enzyme co-factor has hindered their use in continuous sensors [1,4,10]. Because the co-factor serves as an electron acceptor or donor, a replenishing source of co-factor is required.

In our studies, we use the enzyme glutamate dehydrogenase (GDH) to detect and measure glutamate. GDH catalyses the oxidative deamination of glutamate to form α-ketoglutarate. NAD^+ is an obligatory co-factor and serves as the electron acceptor in the reaction. The resulting reduced co-factor, NADH, fluoresces when excited with UV light. The NADH fluorescence as a function of time provides an optical output that is directly related to the glutamate concentration [6].

To create the sensor material, the GDH is encapsulated in a silica sol-gel glass. The silica forms a porous three-dimensional matrix around the enzyme, providing immobilization without covalent attachment [2,5]. The enzyme-doped material is transparent in the UV and visible spectrum making it ideal for spectroscopic measurements. With this method of encapsulation, we see no measurable leaching of enzyme from the gel and the enzyme maintains its activity for many weeks [6].

To create the sensor, GDH doped sol-gel glass is coated onto the tip of an optical fiber. In this form, the sensor functions as a dosimeter, making individual measurements in a sample well that also contains the required co-factor NAD^+. To transform this dosimeter into a continuous sensor, a method of regenerating NAD^+ from NADH is required.

One means of achieving co-factor regeneration is through photochemical reactions. When excited with visible light, the organic dye thionine undergoes a reaction with NADH resulting in relaxed thionine and NAD^+ [7,8]. Because it is the excited form of thionine that reacts with NADH, NAD^+ regeneration only occurs when the sample is irradiated with visible light. By combining enzymatic reactions with thionine excitation, it should be possible to control the regeneration reaction for continuous sensor function.

In this paper, we determine if NADH can be photochemically converted to NAD^+ for use in the oxidative deamination of glutamate by GDH. The effect of thionine concentration on NADH conversion rate is explored. In addition we determine if NADPH can undergo a similar reaction. Finally we observe the effects of sol-gel encapsulation on the rate of NADH conversion.

EXPERIMENTAL

Materials Synthesis

Tetramethoxysilane (TMOS) and thionine acetate were purchased from Fluka. NADH, NAD^+, glutamate dehydrogenase, and glutamate were purchased from Sigma. All materials were used as purchased. All solutions were made with phosphate buffer (0.02 M pH 7.0, 0.02 M NaCl).

Photochemical Regeneration of NAD^+ from NADH

NADH conversion rates in solution were determined by following the NADH fluorescence over time in samples exposed to different concentrations of excited thionine. Thionine was dissolved in phosphate buffer and sonicated for 15 minutes to form a stock solution of 100 μM. The thionine solution was diluted with phosphate buffer and added to 8 wells of a 96 well plate (NUNC N-167008) at varying concentrations. Fluorescence measurements were taken with a Labsystems Fluoroskan Ascent microplate reader. Background fluorescence at 355nm excitation and 460nm emission was taken for 20 counts with a 100 ms integration time. NADH was then injected in the well and an initial NADH fluorescence baseline was measured (40 counts, 100 ms integration time, 355nm excitation, 460 nm

emission). Thionine excited at 584nm and the NADH fluorescence was periodically measured over time. The thionine concentrations after NADH injection were 1μM, 10μM, 20μM, and 50μM each in duplicate. The initial NADH concentration in each well was 100 μM.

To confirm that NAD^+ was produced in the reaction of NADH with excited thionine, we determined if the reaction product could serve as a co-factor in the GDH reaction. GDH (8.8 mg/mL) and NADH (3 mM) solutions were combined in the wells of a 96 well plate (NUNC N-167008) either with or without the addition of thionine (50 μM). Each sample was excited with visible light at 584nm for 3 minutes. Then glutamate (500 μM) was injected into each well and the NADH fluorescence (1000counts, 100 ms integration time, 355nm excitation, 460 emission) was measured for 3 minutes.

Photochemical Regeneration of $NADP^+$ from NADPH in solution and in sol-gel monoliths

TMOS was hydrolyzed under acidic conditions (1.2% by volume 0.04N HCl) and a 1:2 TMOS:water molar ratio. The solution of TMOS, water, and acid was sonicated for 15 minutes. The resulting sol was filtered using 0.2μm HT Tuffryn membrane. NADPH gels were produced by combining 60μL NADPH solution of varying concentration with 40μL hydrolyzed TMOS sol in the well of a microplate. Solution samples were prepared by combining 60μL NADPH solution of varying concentration with 40 μL phosphate buffer. Gel and solution samples with final NADPH concentrations of 33 μM, 66 μM, and 100 μM were produced in triplicate. The samples were aged for 1 hour at room temperature with 20μL of phosphate buffer to prevent drying of the gels. Samples were analyzed using a Fluorskan Ascent microplate fluorometer. At time zero, 20 μL of 60 μM thionine were injected into each sample. Thionine was excited at 584 nm over five intervals of 200 seconds. Thionine fluorescence emission at 620 nm (integration time 100 ms) was collected during these intervals as well. NADPH fluorescence was measured at 460 nm (excitation 355nm, 20 ms integration time, 5 measurements) before and after each thionine excitation interval.

RESULTS AND DISCUSSION

The goal of these experiments was to demonstrate that excited thionine can be used in the sol-gel environment to convert NADH to the oxidized form, NAD^+, that is required for the GDH reaction. It was previously demonstrated that excited thionine reacts with NADH to form NAD^+ and relaxed thionine in solution [7,8]. Our objective is to couple this reaction to the GDH reaction, as illustrated in figure 1, within the sol-gel matrix. In addition, we want to characterize the kinetics of the thionine reaction, so that we can properly design and control the regeneration rate in the functioning fiber optic sensor.

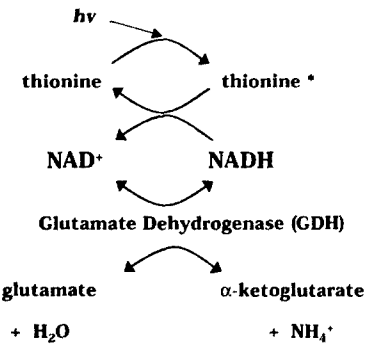

Figure 1: Reaction scheme of NAD^+ regeneration coupled to the GDH reaction. NADH is produced during the oxidation of glutamate. This NADH reacts with excited thionine, thionine*, to form ground state thionine and NAD^+. The regenerated NAD^+ can then serve as the electron acceptor in another round of glutamate oxidation.

Excited thionine reacts with NADH to produce NAD^+ for participation in the GDH reaction.

First, we demonstrate that NADH can be regenerated through a reaction with excited thionine to a form that can be used by GDH. In this experiment samples of GDH and NADH with and without thionine were exposed to light at 584 nm. After exposure, glutamate was injected to each sample well and the NADH fluorescence was measured over time. Figure 2 shows the fluorescence change due to NADH production from the GDH reaction over time.

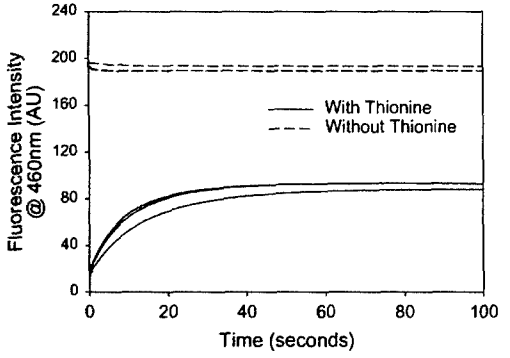

Figure 2: After reaction with excited thionine, NADH is regenerated to a form that is usable by glutamate dehydrogenase. The GDH reaction is measured by the observing NADH fluorescence as a function of time in samples containing GDH and NADH. The samples have been exposed to light in the presence (solid lines) or absence thionine (dashed lines) prior to glutamate injection at t=0. No NADH production is seen in samples without thionine. Samples that did contain thionine show an NADH production due to the enzyme reaction.

Glutamate was injected at t = 0. The samples without thionine show constant NADH fluorescence, corresponding to the concentration of NADH that was initially added to the well. No NAD^+ is present in these sample, so we see no enzymatic NADH production after glutamate is added. Samples containing thionine, however, show a reduced fluorescence at t=0, indicating that the NADH was consumed during the light exposure period. After addition of glutamate to these samples, there is an increase in NADH fluorescence from the GDH reaction. Because NAD^+ is required for the GDH reaction, this observance of GDH activity indicates that NAD^+ was present at t = 0. Since no NAD^+ was added to the well, the initial NADH must have been converted to NAD^+ during the light exposure period. This is consistent with previous findings [9].

This experiment demonstrates that the photochemical oxidation of NADH can be coupled to the GDH reaction for co-factor regeneration purposes. While this experiment was conducted in solution, experience has shown that photochemical reactions that occur in solution also occur in the wet gel [3].

The conversion rate of NADH to NAD^+ is controlled by thionine concentration.

Next we set out to better understand the kinetics of the reaction between thionine and NADH. Samples of NADH in phosphate buffer were exposed to varying concentrations of thionine ranging from 1μM to 50 μM. The thionine was excited at 584 nm and NADH fluorescence measurements were taken periodically to follow the reaction of NADH with excited thionine over time. As seen in figure 3A, the NADH fluorescence disappeared faster in high thionine concentrations. The initial slope of each curve was fit to a line enabling one to determine the initial rate of NADH conversion. Figure 3B shows the initial NADH conversion rate as a function of thionine concentration.

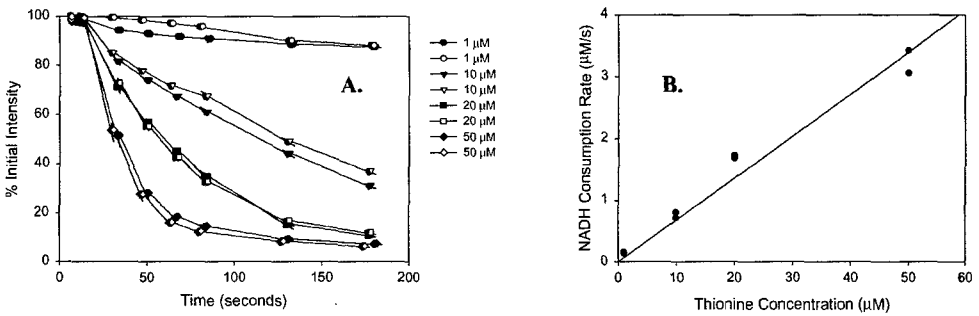

Figure 3: A. NADH fluorescence intensity is measured over time during exposure to excited thionine at varying concentrations. **B.** The initial rate of NADH consumption is a linear function of thionine concentration.

Excited thionine oxidizes NADPH in solution and in the sol-gel environment.

Some dehydrogenases preferentially use NADPH rather than NADH as a co-factor. To determine if thionine reacts with NADPH as well as NADH and to understand the affects of sol-gel encapsulation on the reaction kinetics, we compared the reaction rates of NADPH with thionine in solution with the reaction rates in sol-gel monoliths. Encapsulation did not affect the fluorescence emission spectra of the thionine when excited at 584nm (data not shown). When the samples were exposed to light at 584nm, NADPH conversion was observed in both solution and in the sol-gel. The loss of NADPH fluorescence was similar to the NADH decay in figure 3 and could be fit with a single exponential decay (data not shown). As seen in table I the NADPH decay rate constant, k_{NADPH}, was reduced by a factor of 10 in the sol-gel compared with the solution.

Table I: A comparison of NADH conversion rates in solution and in the sol-gel.

	solution	*sol-gel*
k_{NADPH}	9.4 (±3.3) x 10^{-3} s^{-1}	8.0 (±0.9) x10^{-4} s^{-1}

CONCLUSIONS

We have presented a photochemical method of NAD^+ regeneration by reaction of NADH with excited thionine. Excited thionine reacts with NADH to form NAD^+ that can be used by glutamate dehydrogenase for oxidation of glutamate. The rate of NADH conversion to NAD^+ can be controlled by the thionine concentration. A similar reaction occurs between NADPH and excited thionine in solution and in the sol-gel environment. The reaction rate in bulk sol-gel is about 1 order of magnitude slower than in solution.

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